

# Does the Kunitz domain from the Alzheimer's amyloid $\beta$ protein precursor inhibit a kallikrein responsible for post-translational processing of nerve growth factor precursor?

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Alternative splicing of the Alzheimer's amyloid  $\beta$  protein precursor (ABPP) message leads to the production of several variants of this precursor polypeptide. Two of these variants contain a domain that is highly homologous to members of the Kunitz class of protease inhibitors. In order to initiate a study of the physiological role of this domain, we have produced active ABPP Kunitz inhibitor by constructing and expressing a synthetic gene in *E. coli*. Nerve growth factor (NGF) deficiency has been suggested as a possible cause of the neural degeneration characteristic of Alzheimer's disease, and trypsin and  $\gamma$ -NGF are the two enzymes that have been shown to be capable of processing  $\beta$ -NGF precursor to active, mature  $\beta$ -NGF in vitro, therefore the specificity of purified recombinant ABPP Kunitz inhibitor was analyzed with respect to these two proteases. Binding of isolated ABPP Kunitz domain both to trypsin ( $K_{i,app} < 10$  nM and to  $\gamma$ -NGF ( $K_{i,app} = 300$  nM) was observed. This difference in binding to the two proteases correlates with the approximately 20-fold higher rate observed for in vitro processing of the  $\beta$ -NGF precursor by trypsin compared to processing by  $\gamma$ -NGF, indicating that perhaps the inhibitor mimics the interaction of the  $\beta$ -NGF precursor with proteases. The kallikrein actually responsible for  $\beta$ -NGF precursor processing in vivo is unknown, but these results suggest that it is capable of being significantly inhibited by exposure to the ABPP Kunitz domain.

Alzheimer's disease; Amyloid  $\beta$  protein; Kunitz inhibitor; Kallikrein; Nerve growth factor

## 1. INTRODUCTION

Alzheimer's disease and trisomy 21 (Down's syndrome) are almost invariably associated with the deposition of neuritic plaque in the brains of stricken individuals. The precursor of the major polypeptide found in plaque, amyloid  $\beta$  protein, has recently been characterized by cDNA cloning [1–3]. Several different mRNAs exist, all splicing variants of a transcript derived from a single gene, and these appear to encode precursors 695, 751 and 770 amino acids in length [1–3]. The messages encoding the two larger precursors have an additional exon spliced into the interior of the coding region, and the translated sequence of this exon suggests that it encodes a protein domain that is a member of the Kunitz class of protease inhibitors [4]. Consistent with this prediction, trypsin inhibitory activity was found in conditioned medium from cells transfected with amyloid  $\beta$  protein precursor (ABPP) cDNA containing the inhibitor sequences [3,5]. The Kunitz inhibitor domain from ABPP has been highly conserved

in evolution [6,7], and thus it is likely that this domain plays a functionally significant role in vivo. Based on the known properties of the Kunitz inhibitor family, this role almost certainly involves stoichiometric binding to the active site of some endogenous protease or esterase [4].

Nerve growth factor (NGF), NGF mRNA, NGF receptors, as well as NGF-related neurotrophic factors, have been found in the brains of adult mammals, suggesting that NGF-like molecules play a physiologically significant role in normal adult brain [8–11]. Exogenously administered NGF has also been shown to be capable of preventing degeneration of cholinergic neurons and associated memory loss in adult animals with experimentally induced lesions [12]. Because Alzheimer's disease is associated with neuronal cell loss, including those in the ascending cholinergic pathways of the basal forebrain [8], and because intraventricular administration of anti-NGF antisera in experimental animals produces Alzheimer's-like histological changes in the brain [13], it has been hypothesized that NGF deficiency may be causally related to the pathogenesis of Alzheimer's disease [8].

The active neurotrophic factor,  $\beta$ -NGF, is produced from relatively inactive precursor via proteolytic

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processing [14–16]. Thus the production of endogenous NGF activity in the brain presumably depends on the action of a kallikrein on the  $\beta$ -NGF precursor, and disruption of this post-translational processing step should result in a decrease in active NGF. It is conceivable that a pathological state, manifested by poor neuron viability, could result from inappropriate expression or cell localization of the amyloid Kunitz inhibitor domain if this caused it to inhibit the processing enzyme for  $\beta$ -NGF activation. Similar proteolytic processing also appears to be necessary for the maturation of other NGF-like neurotrophic factors [9–11], so the actions of these should likewise be susceptible to interference by this mechanism.

In order to begin testing the feasibility of this hypothesis we have prepared active ABPP Kunitz domain inhibitor by expressing a synthetic coding sequence for the inhibitor using an *E. coli* secretion vector. We present here the results of binding studies with this isolated inhibitor domain and the two proteases, trypsin and  $\gamma$ -NGF, that have been shown to be capable of correctly processing the  $\beta$ -NGF precursor in vitro [14,15].

## 2. MATERIALS AND METHODS

### 2.1. Synthetic Kunitz inhibitor coding sequences

Eight oligodeoxynucleotides (Fig. 1A), representing the duplex coding sequence for the Kunitz domain of the Alzheimer's amyloid  $\beta$  protein precursor (codons 287–344 according to the numbering of [1]), were synthesized by the deoxynucleoside H-phosphonate method [17]. Codon choice was governed by an avoidance of codons known to be poorly expressed in *E. coli* [18] or codons that would result in strong secondary structural features (e.g. hairpins) in the synthetic gene transcript. In addition, the oligonucleotides were designed to yield a *MluI* site just upstream of the Kunitz inhibitor coding sequence and tandem stop codons followed by a *SalI* site at the 3'-end of the coding sequence (Fig. 1A).

### 2.2. Expression of the synthetic Kunitz inhibitor gene

*EcoRI/MluI* fragment containing the alkaline phosphatase promoter a ribosome binding site, and the STII signal sequence [19–21] was isolated from pST2A. Plasmid pST2A (kindly provided by D. Henner, Genentech) is a derivative of pHGH4R [21] in which codons 20–22 of the STII signal sequence, AAT-GCC-TAT (Asn-Ala-Tyr) [19,20], were modified by site-directed mutagenesis to introduce a unique *MluI* site, AAC-GCG-TAT. A 4.2 kbp *EcoRI/SalI* fragment was isolated from the vector p651 (C.B. Marks, unpublished); this fragment is identical in sequence to the 3.7 kbp *EcoRI/SalI* fragment from pBR322, except for a 0.45 kbp *RsaI/AhaII* fragment containing the phage f1 + strand origin [22] inserted at the pBR322 *PvuII* site. Two pmols of each of the oligonucleotides comprising the synthetic ABPP Kunitz domain coding sequence were phosphorylated with rATP and T4 polynucleotide kinase in 70 mM Tris-HCl (pH 7.8), 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, then mixed, heated to 95°C, and cooled slowly to anneal the strands. The annealed oligonucleotides were mixed with the 0.52 kbp *EcoRI/MluI* fragment from pST2A and the 4.2 kbp *EcoRI/SalI* fragment from p651, and these were joined by incubating them in the same buffer as above with rATP and T4 DNA ligase. The ligation mixture was used to transform MM294 cells and random colonies were picked for preparation of single-stranded plasmid DNA using the M13KO7 helper phage [23]. DNA isolates were screened for the presence of the inserted synthetic DNA by

dideoxy DNA sequencing [24] and one isolate, termed pSalz1 (Fig. 1B), was chosen for further characterization. pSalz1 was checked by dideoxy DNA sequencing of both strands to ensure that the inserted synthetic DNA sequence was correct. *E. coli* W3110*tonAtolA*, transformed with pSalz1, was grown in low phosphate medium as described previously [21].

### 2.3. Purification and characterization of recombinant ABPP Kunitz inhibitor domain

Initial purification of the ABPP Kunitz inhibitor domain was carried out as previously described for *E. coli*-derived bovine pancreatic trypsin inhibitor (BPTI) [25,26], except that the acid precipitation step was performed with one-percent trichloroacetic acid, and elution of the inhibitor from the trypsin-Sepharose affinity column was with 10 mM HCl, 0.5 M KCl. Final purification was by Mono Q FPLC anion exchange chromatography with a 0.0–0.25 M gradient of NaCl in 20 mM Tris (pH 8.0), or by Superose 12 gel filtration chromatography in 150 mM NH<sub>4</sub>HCO<sub>3</sub>. A sample of the purified inhibitor was subjected to 31 cycles of Edman degradation on an Applied Biosystems 470A Protein Sequencer. The N-terminal sequence deduced was VREVSXSEQAETGPXRAMISRWYFDVTEGKXA. Amino acid analyses were performed using the method of Meltzer et al. [27] on independent samples purified by Mono Q anion exchange and Superose 12 gel filtration chromatography. The following composition, averaged from determinations on two independently purified samples, was found (theoretical values in parentheses): Asx, 5.02 ± 0.07 (5); Ser, 2.63 ± 0.03 (3); Gly, 7.39 ± 0.08 (7); Glx, 6.70 ± 0.36 (7); Thr, 2.88 ± 0.02 (3); Ala, 5.29 ± 0.06 (5) Val, 4.01 ± 0.14 (4); Met, 1.74 ± 0.05 (2); Tyr, 3.13 ± 0.02 (3); Ile, 0.98 ± 0.03 (1); Leu, 0.0 (0); Phe, 4.20 ± 0.07 (4); His, 0.0 (0); Trp, 0.85 ± 0.05 (1); Lys, 1.00 ± 0.08 (1); Arg, 4.23 ± 0.04 (4); Pro, ND (2); Cys, ND (6) (ND = not determined).

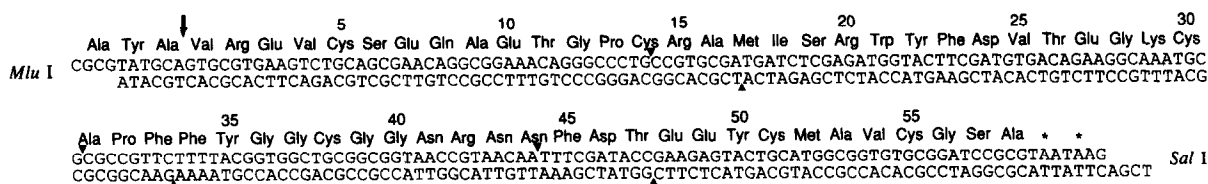
### 2.4. Inhibition studies

The molar concentration of purified recombinant ABPP Kunitz domain inhibitor in a reference solution was determined by quantitative amino acid analysis, assuming a molecular mass of 6415 Da. BPTI (a gift from Miles Laboratories) was quantitated based on the published extinction coefficient at 280 nm of 0.84 for a 1 mg/ml solution [28]. Trypsin was purchased from Boehringer-Mannheim. Murine 7S NGF (Sigma) was activated by incubating it for 24 h at 5°C in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA. Activating 7S NGF in this manner uncovers the  $\gamma$ -subunit esteropeptidase activity, resulting in a reactivity towards substrates and protease inhibitors that is indistinguishable from the reactivity of the purified  $\gamma$ -subunit [29–31]. Trypsin and  $\gamma$ -NGF activities were quantitated using the burst titrant *p*-nitrophenyl *p*'-guanidinobenzoate [32,33]. Aliquots containing 0.1025  $\mu$ M of enzyme were incubated for at least 15 min in 50 mM Tris-HCl (pH 7.4) with various concentrations of the purified ABPP Kunitz domain or BPTI, then 25  $\mu$ l of 16 mg/ml *N*- $\alpha$ -benzoyl-DL-arginine-*p*-nitroanilide was added and the change in absorbance at 405 nm was monitored at 25°C as a function of time. Reaction volumes were 1 ml.

## 3. RESULTS

Oligonucleotides representing codons 287–344 of ABPP<sub>751</sub> [1] were ligated together and fused to a gene fragment encoding the heat-stable enterotoxin II (STII) signal sequence in an *E. coli* secretion vector (Fig. 1). This was done in such a way as to place the ABPP Val-287 residue (corresponding to residue one of the Kunitz inhibitor homologue, bovine pancreatic trypsin inhibitor (BPTI) [4,28]) on the C-terminal side of the signal peptidase cleavage site; tandem TAA stop codons were placed after the Ala-344 residue (Fig. 1). When ex-

A



B

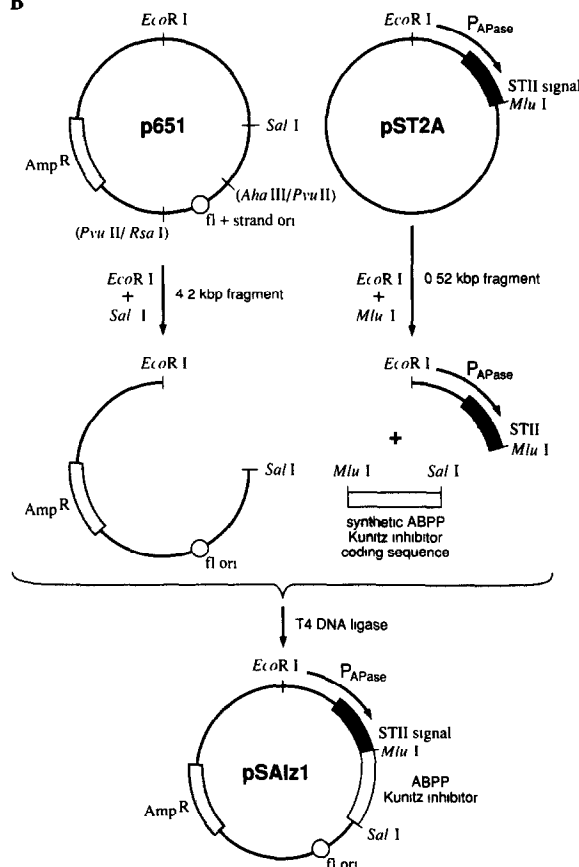


Fig. 1. Expression of the ABPP Kunitz inhibitor domain. (A) Shown is the construction of the synthetic duplex coding sequence for the ABPP Kunitz inhibitor domain. The numbering is based on that of the homologous inhibitor, BPTI [25,28], and starts with the residue corresponding to Val-287 of ABPP<sub>751</sub> [1]. The sites where oligonucleotides were joined in the ligation reaction are indicated by filled triangles. The signal peptidase cleavage site between the STII leader peptide and the Kunitz inhibitor sequences is indicated by a vertical arrow. Also shown are the *Mlu*I and *Sal*I hemi-sites at the termini of the DNA fragment. (B) The expression vector, pSAIz1, was constructed via a three-way ligation of the synthetic *Mlu*I/*Sal*I fragment, an *Eco*RI/*Mlu*I fragment containing the alkaline phosphatase promoter and the STII signal peptide coding sequences, and an *Eco*RI/*Sal*I fragment containing the plasmid and f1 phage replication origins and the  $\beta$ -lactamase gene from plasmid pBR322.

pressed in *E. coli* this construct produced detectable levels of secreted trypsin inhibitory activity. This activity was purified to homogeneity from the culture medium by trichloroacetic acid precipitation, trypsin-Sepharose affinity chromatography, and Mono Q anion exchange or Superose 12 gel filtration FPLC [25,26]. Amino acid analysis of the purified material yielded results consistent with the predicted composition of the ABPP Kunitz inhibitor, and 31 cycles of N-terminal amino acid sequencing yielded the predicted

sequence and showed that no detectable internal clips were present. The disulfide bonding pattern for the recombinant ABPP Kunitz inhibitor was not determined. However, an X-ray crystallographic analysis of the ABPP Kunitz inhibitor tertiary structure (R. Hynes and A. Kosiakoff, personal communication) showed that the disulfides were identical to the Cys5-Cys55/Cys14-Cys38/Cys30-Cys51 pattern observed in natural BPTI as well as in *E. coli*-derived BPTI produced by a similar system ([25, 28] C.B.M., B.N. and S.A., unpublished).

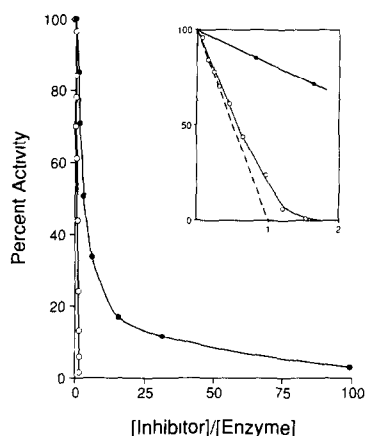


Fig. 2. Inhibition of  $\gamma$ -NGF and trypsin by recombinant ABPP Kunitz domain. The  $\gamma$ -NGF esterase of 7S murine NGF (activated as described in section 2) or bovine trypsin were incubated with various concentrations of the ABPP Kunitz inhibitor domain at a constant enzyme concentration of  $0.1025 \mu\text{M}$ . The activity of unbound enzyme was monitored by measuring the change in absorbance at 405 nm due to BAPA hydrolysis as a function of time (see section 2). The percent residual activity is graphed as a function of the inhibitor:enzyme ratio; the closed symbols represent the data for  $\gamma$ -NGF and the open symbols the data for trypsin. The insert shows the data at low inhibitor:enzyme ratios with the dashed line indicating the theoretical inhibition curve that would be produced by an inhibitor binding stoichiometrically to the enzyme with infinitely high affinity [36]. Apparent inhibition constants ( $\gamma$ -NGF,  $K_{i,\text{app}} = 3.0 \times 10^{-7} \text{ M}$ ; trypsin,  $K_{i,\text{app}} < 1.0 \times 10^{-8} \text{ M}$ ) were derived from these data using the equation  $[I]/(1-a) = K_{i,\text{app}}(1/a) + [E]$ , where  $[I]$  is the inhibitor concentration,  $[E]$  the enzyme concentration, and  $a$  the residual activity [36,37] or by the method of Green and Work [38]. Similar binding experiments performed on  $\gamma$ -NGF and trypsin with BPTI (not shown) yielded results consistent with published inhibition data for these complexes [28,31].

In the murine 7S NGF complex the  $\gamma$ -subunit is inactive because its active site is complexed with the C-terminal arginine of the  $\beta$ -subunit [34]. To obtain free  $\gamma$ -NGF the complex was dissociated in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA [29–31]. Aliquots of this were incubated with different concentrations of the purified ABPP Kunitz inhibitor, then added to *N*- $\alpha$ -benzoyl-DL-arginine-*p*-nitroanilide. The proportion of free enzyme was determined by a spectrophotometric determination of the relative ester hydrolysis rate. Esterase activity decreased with increasing amounts of inhibitor (Fig. 2). The apparent dissociation constant for  $\gamma$ -NGF and the ABPP Kunitz inhibitor was determined from these data to be approximately 300 nM [35–37].

Similar inhibition experiments with trypsin indicated that the ABPP Kunitz domain bound to this enzyme with an affinity significantly higher than its affinity for  $\gamma$ -NGF (Fig. 2). The apparent dissociation constant for trypsin and the ABPP Kunitz inhibitor was estimated by the method of Green and Work [38] to be less than 10 nM.

#### 4. DISCUSSION

The etiology of Alzheimer's disease is obscure. Research efforts in this area have primarily focussed on the phenomenon of neuritic plaque deposition [39,40] and on the possible role of trophic factors, e.g. nerve growth factor (NGF), in preventing the neuropathological changes observed [8]. Some linkage between these two approaches has been suggested by the recent cDNA cloning of the precursor for the amyloid  $\beta$  protein [1–3], the major polypeptide component of neuritic plaque. The sequence of this amyloid  $\beta$  protein precursor (ABPP) has led to the discovery of several new bioactive protein fragments, some of which have trophic [41,42] or neurocytopathic [43] effects. The ABPP clones also reveal that the transcript for the precursor is alternatively spliced, resulting in at least two message species with an additional exon in the coding region. The amino acid sequence of this alternatively spliced exon is homologous to members of the Kunitz class of protease inhibitors [1–4].

In this paper we have shown that the ABPP Kunitz inhibitor binds to and inhibits the two proteases, trypsin and  $\gamma$ -NGF, that have been shown to be capable of correctly processing the inactive  $\beta$ -NGF precursor in vitro [14,15]. Inhibition of trypsin by the ABPP Kunitz domain is strong, and inhibition of  $\gamma$ -NGF relatively weak although still significant (Fig. 2). This inhibition spectrum correlates well with the relative efficiencies with which these two proteases process the  $\beta$ -NGF precursor: trypsin is approximately 20-fold more active than  $\gamma$ -NGF in this reaction [14]. Thus, the specificity of the ABPP Kunitz domain may reflect the structure of the  $\beta$ -NGF precursor, with the inhibitor:protease complex mimicking the substrate:enzyme interaction of pro $\beta$ -NGF with kallikrein(s).

The ABPP Kunitz domain may function normally as part of a feedback control mechanism that modulates the production of active NGF and other growth factors (Fig. 3). It has been reported that high levels of inhibitor-containing ABPP restrict cell growth when added to the medium of ABPP-deficient fibroblasts, while the same levels of ABPP lacking the inhibitor domain do not exhibit this effect [42].

Whether the ABPP Kunitz inhibitor actually inhibits the kallikrein-mediated processing of  $\beta$ -NGF precursor in vivo is unknown. The  $\beta$ -NGF precursor expressed via a vaccinia virus vector in a variety of cell types is processed to mature NGF prior to secretion [16]. This indicates not only that the processing enzymes are unlikely to be cell-specific, but also that the processing occurs intracellularly in secretory granules or in the Golgi apparatus. This compartmentalization may prevent free, extracellular ABPP Kunitz inhibitor from contacting the processing enzymes. However, in cells synthesizing both ABPP and growth factor, the Kunitz domain could down-regulate growth factor maturation

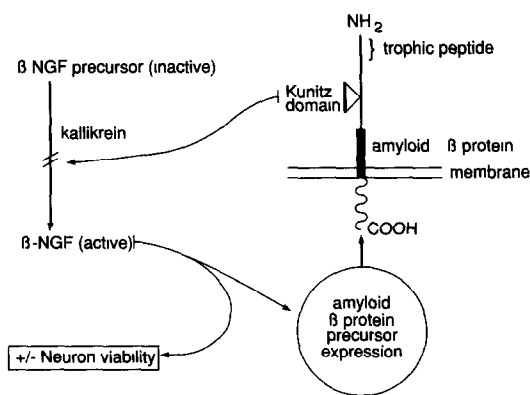


Fig. 3. Model for how the Kunitz domain could modulate production of active neurotrophins in the brain. ABPP is shown as a membrane-associated polypeptide (adapted from [3]) although it also is found as a secreted protein [5,44–46]. The amyloid  $\beta$  protein (A $\beta$ ) peptide is indicated by cross-hatching, and the N-terminal neurotrophic peptide [41] and the alternatively spliced Kunitz domain are also shown. The example given is for a negative-feedback loop regulating  $\beta$ -NGF production. In addition to modulating nerve cell viability,  $\beta$ -NGF increases ABPP gene expression [53]. The increased levels of the amyloid  $\beta$  protein precursor may then down-regulate  $\beta$ -NGF maturation if the alternatively spliced Kunitz inhibitor domain is present and binds to the kallikrein(s) that proteolytically process the growth factor precursor.

if it were sorted to the same compartment as the growth factor precursor.

Conceivably, secreted ABPP [5,44–46] could also inhibit growth factor maturation by binding to specific cell-surface receptors via the N-terminal domain (Fig. 3) and being transported to the processing site of the growth factor precursor after internalization. Resialylation of asialo cell surface receptors has demonstrated that there are pathways in the cell which allow traffic of membrane proteins from the plasma membrane to the Golgi [47,48].

Recently, Oltersdorf et al. [5] and Van Nostrand et al. [49] have reported that the Kunitz domain-containing ABPP was identical to protease nexin II, a protease inhibitor secreted from fibroblasts [50]. Protease nexin II has been shown to bind to trypsin,  $\gamma$ -NGF, EGF binding protein, and chymotrypsin [49, 50], suggesting that the results we have obtained with the isolated ABPP Kunitz domain also apply to the behavior of the Kunitz domain in the context of the intact amyloid  $\beta$  protein precursor. Van Nostrand and Cunningham [50] have reported that protease nexin II does not form a stable complex with plasma kallikrein or plasmin, both of which bind to BPTI [28]. This indicates that the specificity of the ABPP Kunitz inhibitor may be greater than that of BPTI, consistent with its proposed role as an inhibitor of specific growth factor processing enzymes. It also suggests that the ABPP Kunitz inhibitor could be used as an affinity ligand for the purification and characterization of such enzymes.

Recently a high resolution three-dimensional structure has been determined for the ABPP Kunitz inhibitor by X-ray crystallography (R. Hynes and A. Kossiakoff, personal communication). One striking difference between the ABPP Kunitz domain structure and the BPTI structure is in the region of residues 38–40 where the backbone adopts a different conformation in the two molecules. The sequence at these residues, Cys-Gly-Gly (Fig. 1), in the ABPP Kunitz inhibitor allows it to adopt the alternative backbone fold, and this region overlaps the reactive site and specificity determining residues of the inhibitor [28]. It is intriguing that this same sequence motif commonly occurs in the dendrotoxins, which are neurotoxic members of the Kunitz inhibitor family isolated from mamba venoms [51,52].

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